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BLOOD TYPES IN PACIFIC SALMON

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SPECIAL NOTE

The International North Pacific Fisheries Commission, established in 1953 by the International Convention for the High Seas Fisheries of the North Pacific Ocean, coordinates the research of the member nations: Japan, Canada, and the United States. The resulting investigations provide data to the Commission for use in carrying out its duties in connection with fishery conservation problems in the North Pacific Ocean. Publication of this scientific report has been approved by the United States Section of the Commission.

United States Department of the Interior, Fred A. Seaton, Secretary
Fish and Wildlife Service, Arnie J. Suomela, Commissioner

BLOOD TYPES IN PACIFIC SALMON

by

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Contribution No. 12 to research conducted with the approval of the United States Section of the International North Pacific Fisheries Commission.

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ABSTRACT

Intraspecific differences in erythrocyte antigens (blood types) were shown to occur in four species of Pacific salmon, the sockeye or red salmon (Oncorhynchus nerka), the chinook or king salmon (O. tshawytscha), the chum salmon (O. keta), and the pink salmon (O. gorbuscha). Antisalmon-erythrocyte sera prepared in rabbits and chickens were used after absorption of species-specific antibodies. Some of these blood types were shown to differ in their frequency of occurrence between different geographic races. In addition, isoimmunizations were conducted on one race of sockeye salmon. Antisera of seven different specificities were prepared and at least eight different patterns of antigenic composition were displayed by the cells tested.

These results indicate that considerable antigenic diversity exists in salmon. Reagents to detect valuable markers for the investigation of geographic races of salmon should be obtained through further research.

INTRODUCTION

One of the most important problems in fishery biology is recognition of "races", or reproductively isolated subpopulations, of fishes. Since there may be little or no interbreeding or recruitment between such populations, successful regulation or management of fishery resources must be conducted in such a way as to recognize their independence.

Present methods for distinguishing between the races of a particular species of fish are based on tagging by various means, or by searching for morphometric or meristic differences between the races (Rounsefell and Everhart 1953).

It has been shown with many animals (Mayr 1942, Dobzhansky 1951), including fishes (Gordon 1947), that reproductively isolated subpopulations of species differ in the frequencies of one or more variable genes. Therefore, a very useful adjunct to the methods of racial identification, now used in fishery management and research, would be the recognition and definition of genetically controlled polymorphic characters. One class of such characters which has been found in every animal adequately investigated is intraspecific antigenic variations in the red blood cells. These characters are more simply known as blood types.

Landsteiner (1900) was the first to demonstrate the occurrence of blood types. He found them in man with natural isoagglutinins. In the same year Erlich and Morganroth (1900) demonstrated blood types in goats using immune isohemolysins induced by transfusions. Subsequently, many additional instances of intraspecific antigenic differences have been demonstrated. The results are most notable in the case of man (Race and Sanger 1954), cattle (Stormont et al. 1951) and chickens (Briles et al. 1950).

In each of these species, blood types have been used to study reproductively isolated populations. Thus, blood type frequencies have been used to characterize

races of men (Boyd 1950, Mourant 1954), breeds of cattle (Owen et al. 1947) and inbred lines of chickens (Schultz and Briles 1953).

The studies of Fujino (1958) demonstrating the occurrence of blood types in whales are of particular interest to marine biologists.

Until recently there have been relatively few published studies concerning the possible existence of blood types in fishes. Some of these demonstrated marked interspecific differences in cellular antigens but provided little or no evidence for intraspecific heterogeneity of such antigens. Noguchi (1903 a, b) found that the serum of several species of fishes would agglutinate the red blood cells of other species but no intraspecific differences were noted. He did produce isoagglutinins and isohemolysins in two species of turtle, Chrysemys picta and Chelopus guttatus, demonstrating that cold blooded vertebrates may possess intraspecific antigenic differences. Tóth (1932) found no evidence for the existence of blood types in carp from 280 crossmatches. Jensen (1937) tested for natural isoagglutinins in the cod (Gadus morrhua) and also attempted to demonstrate individual differences with the serum of a single rabbit immunized with the red cells of an individual cod. He did not find any conclusive evidence for the existence of blood groups in cod.

Suyehiro (1949) reviewed the previous Japanese literature in which there were no instances of the demonstration of blood types in fishes. He was evidently the first to demonstrate individual antigenic differences in fish blood cells since he found a few instances of natural isoagglutinins in the eel (Anguilla japonica) and the gilthead (Sparus swinhonus Gunther). In 249 crossmatches of blood cells and serum from cod (Gadus macrocephalus) he found no evidence for natural isoagglutinins. Suyehiro also tested blood samples from 336 fishes of 30 different species with human ABO blood typing sera and found that only 91 were agglutinated. In 21 of the 30 species, individuals varied in their reactivity to the human sera tested.

Cushing and Sprague (1953) studied the agglutinative activity of human anti-A and anti-B sera and rabbit antisheep cell serum for the erythrocytes of a number of species of fish. Considerable antigenic diversity was noted between species but no individual differences were found within the members of the species tested.

Recent reports have indicated a major breakthrough in the efforts to discover blood types in fishes. Hildemann (1956) applied the method of isoimmunization, which has been so successful in detecting blood types in other animals, to goldfish (Carassius auratus). One of the isoimmune sera detected six different antigenic types of goldfish. Immune rabbit sera were also prepared by Hildemann, one of which detected a single antigenic difference after careful absorption. Cushing (1956) reported the existence of individual antigenic differences in the oceanic skipjack (Katsuwonus pelamis Linnaeus) detectable with natural isoagglutinins, normal bovine serum, and the sera of rabbits immunized with the whole blood of oceanic skipjack, albacore (Germo alalunga Gemlin), or Pacific mackerel (Pneumatophorus japonicus diego Ayres). Cushing and Durall (1957) discovered and analyzed a natural isoagglutinin system in the brown bullhead (Ictalurus n. nebulosus Le Sueur). This system was found to be analogous to the human ABO system in that four antigenic types were found (i.e., some fish possessed antigen 1, some antigen 2, some both antigens, and some had neither), and when an antigen was lacking, its corresponding isoagglutinin was always present. Ridgway, Cushing, and Durall (1958) found quantitative differences in the reactivity of the cells of individual sockeye salmon (Oncorhynchus nerka Walbaum) with natural antibodies from pig sera and demonstrated that there were significant differences between geographically separated populations in the frequency of the different types detected.

Suzuki et al. (1958) have demonstrated the existence of blood groups in species of tunas and have presented evidence which suggests that differences exist between the blood type frequencies of albacore from the Pacific and Indian Oceans.

The present report provides further evidence for the existence of blood types in Pacific salmons and additional evidence that some of these characters differ in their frequency of occurrence in different races of the same species.

MATERIALS AND METHODS

Most blood samples were taken in the field by severing the caudal artery with a sharp knife and collecting the spurting blood in sterile bottles. Some of the samples, including those used in isoimmunizations, were taken by cardiac puncture. The clotted samples were maintained on ice or in a refrigerator until used. Samples excessively hemolysed or over ten days old were not used for testing. Blood cells from samples as old as three weeks were used for animal inoculation. Cells for testing were washed three times in 10 to 50 volumes of modified Alsever's solution (Bukantz et al. 1946) and adjusted to a 2-percent concentration in this solution. The use of the Alsever's solution as a suspending medium was found to be essential since salmon red blood cells lysed in saline or phosphate-buffered saline solutions.

Most antisera were prepared by giving intravenous or intraperitoneal injections of 0.5 cc. of a 50 percent suspension of washed cells. Such injections were given to rabbits and salmon three times a week for three weeks and to chickens every three days for three or four injections. Four to twelve days after the last injection the animal was bled and serum collected. In most cases, in order to detect individual differences, several additional stimulations at intervals or two weeks to a month were required. Some rabbits also received subcutaneous inoculations of washed suspensions of particulate material from lysed red cells in Freund's adjuvant. The sera were preserved by freezing and stored at -30° C. Serum dilutions were made with phosphate-buffered saline or 1 percent saline solutions.

Absorptions were performed by mixing a 1/2, 1/5 or 1/10 dilution of the heat-inactivated antiserum with an equal volume of washed packed cells, incubating for one hour at room temperature or in the refrigerator, centrifuging the cells down and decanting the absorbed serum. Usually more than one absorption was required to remove all of the antibody present which would react with the absorbing cells.

Tests were performed by mixing 0.1 ml. of absorbed serum, diluted if necessary, with 0.1 ml. of 2 percent cell suspension in Alsever's solution in 10 x 75 mm. tubes. Dilutions are expressed as the final dilution of the original serum, taking into account the dilution by the red cell suspensions. Readings were made after suitable incubation periods at room temperature and usually after overnight incubation in the cold. The settling pattern was judged either smooth (S) or rough (R) and the degree of agglutination scored as 0, ±, 1 plus, 2 plus, 3 plus and 4 plus. In order for a test to be considered, the saline control had to be smooth and negative.

Table 1.--Individual antigenic differences demonstrated by agglutinin absorption tests in the erythrocytes of sockeye salmon from Cultus Lake and Adams River.

(Rabbit antisockeye salmon-erythrocyte serum R19 absorbed and cross-tested with the cells of individual salmon)

Absorbing cells	Test cells: Cultus Lake 1	Cultus Lake 15	Cultus Lake 19	Cultus Lake 12	Cultus Lake 13	Adams River 15	Adams River 11	Adams River 9	Adams River 13	Adams River 8
Cultus Lake 1	0	+	0	+	0	+	+	+	+	+
Cultus Lake 15	+	0	+	+	+	+	+	+	+	+
Cultus Lake 19	+	+	0	+	0	+	+	+	+	+
Adams River 15	0	0	0	+	0	0	0	0	+	0
Adams River 11	0	0	0	0	0	0	0	0	0	0
Adams River 9	0	0	0	+	0	0	0	0	0	0
Adams River 13	0	0	0	0	0	0	0	0	0	0
Saline control	0	0	0	0	0	0	0	0	0	0

RESULTS

The major part of our effort has been directed toward finding blood types in red or sockeye salmon (Oncorhynchus nerka Walbaum). Toward this end we have immunized 26 rabbits and 20 chickens with the washed erythrocytes or stroma from members of this species. For each of the other species of Pacific salmon we have made 2 - 8 rabbit and chicken anti-erythrocyte sera. Individuals or pools from individuals from the same area were used. These antisera were absorbed with the erythrocytes of individual salmon from other areas and the resulting sera tested for residual activity for the cells of a number of individuals. With most antisera produced, numerous absorptions did not reveal any evidence for antigenic heterogeneity within this species. However, blood group differences were demonstrated with a few rabbit immune sera. One of these sera (R19) was analyzed by absorption with the cells of sockeye salmon collected in 1955 from Cultus Lake and the Adams River,

Table 2.--Serological differences between races of Fraser River sockeye salmon.

Area (test cells)	Number tested	Number reacting with reagents prepared by absorbing rabbit R19 serum with cells of: Cultus Lake 19	Cultus Lake 1	Cultus Lake 15	Number not reacting with any of the three reagents
Cultus Lake	17	3	2	12	4
Adams River	17	11	17	16	0
Chi-square		7.6*	56.6*	3.3	4.5*

* Significantly different at the 95 percent level.

both tributary areas on the Fraser River in British Columbia (table 1). The three samples tested from the Adams River race removed all or nearly all of the antibodies present in the serum. The three samples from the Cultus Lake race each removed all of the antibodies specific for their antigens, but left antibodies which would react with antigens present on the cells of other individual tested. Thus, three reagents of different specificites were produced.

These results as well as additional tests made on samples collected from these areas in 1956 with the above absorbed sera are combined in table 2. Statistically significant differences in the proportion of individuals reacting positively with these reagents are apparent for the two populations.

Some of the antisalmon red blood cell sera, prepared in rabbits and chickens, were useful in demonstrating blood group differences in species of salmon other than the one used for immunization. This is somewhat analogous to the demonstration of the Rh blood groups in humans by Landsteiner and Weiner (1941), through the use of antirhesus monkey red blood cell sera.

The evidence for blood groups in chinook or king salmon (O. tshawytscha) is presented in table 3. Absorption of serum R13, an antisockeye salmon red blood cell serum, by the cells of four different chinook salmon from the Columbia River, resulted in the production of reagents of four different specificities. The results indicate a characteristic is present in number one which is absent from the other three. Superimposed on this, there appears to be a kind of subtype difference between 2, 3, and 4 which is shared with 1.

Evidence for blood groups in chum salmon (O. keta) is presented in tables 4 and 5. The experiments outlined in these tables were performed with samples from the Samish River in Washington State. Evidence for the presence of a considerable amount of antigenic heterogeneity in this species and this race is provided by the results presented in table 4, since reagents of four different specificities were obtained by five different absorptions.

Five patterns of reactivity were displayed by the samples tested; numbers 1, 6, 8, and 9 reacted with the sera absorbed by 2, 4, and 5; numbers 2 and 7 reacted with

Table 3.--Demonstration of individual antigenic differences in chinook salmon erythrocytes using rabbit antisockeye salmon serum (Serum R13 absorbed and cross-tested with four individual samples of chinook erythrocytes).

Absorbing cell sample numbers	Test cell sample numbers	Dilutions of absorbed sera 1:10	1:20	1:40	1:80
1(a)	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
2	1	++++	0	0	0
	2	±	0	0	0
	3	0	0	0	0
	4	0	0	0	0
3	1	++++	++	0	0
	2	+	±	0	0
	3	0	0	0	0
	4	0	0	0	0
4(b)	1	++++	++++	+++	+
	2	++++	+++	0	0
	3	++++	+++	++	0
	4	+	0	0	0

(a) This absorbed serum tested against the cells of an additional 12 chinook salmon, none of which reacted. Positive reactions were obtained with the cells of 12 different sockeye salmon.

(b) This absorbed serum tested against the cells of 24 additional chinook salmon. Definite reactions occurred with 16 of them.

the sera resulting from absorption by 1 and 5; number 4 reacted with the sera resulting from absorption with 1, 3, and 5; number 3 reacted with sera from absorption with numbers 1, 2, 4, and 5; and number 5 reacted with the sera from absorption with numbers 2 and 4. The results obtained with individuals 2, 4, and 7 demonstrate some of the complexities involved in the antigenic differences between individuals of a single species. All gave the same reaction with the reagents prepared, except for the weak reaction of the cells of individual 4 with the reagent prepared by absorption with the cells of individual 3. Consistent with this is the fact that reagents of identical specificity were prepared by absorption with cells of individuals 2 and 4. In contrast, absorption by cells of individual 7 removed the antibodies reactive with the cells of all individuals tested, but the cells of individual 7 failed to react detectably in agglutination tests with reagents prepared by absorption with cells of individuals 2, 3, or 4. Such apparent deviations from the principles upon which absorption analyses are generally assumed to rest have been associated, in other species, with the multiple-allelic control of sets of related but nonidentical specificities. The large amounts of cells required to absorb the antibodies present in this serum which would agglutinate the cells of all members of the species, precluded further investigation of this system.

Table 4.--Demonstration of blood types in chum salmon using chicken antichinook salmon serum

(Serum 202 absorbed and tested with cells of individual chum salmon)

Absorbing cell sample numbers	Test cell sample numbers								
	1	2	3	4	5	6	7	8	9
1	0	+	+	++	0	0	+	0	0
2	++	0	++	0	++++	++	0	++++	++
3	0	0	0	+	0	0	0	0	0
4	+++	0	++	0	+++	++	0	+++	+++
5	+++	++++	++	++++	0	++	+++	+	+
7	0	0	0	0	0	0	0	0	0

The results presented in table 5 are less complicated with apparently a single difference detected between chum salmon sample 18 and the others tested. Superimposed on this however are rather marked differences in the strength of reactivity of the other cells tested which may indicate additional antigenic heterogeneity.

Evidence for the existence of blood group differences in pink salmon (O. gorbuscha) is presented in table 6. A single difference was noted which appeared to be correlated with the area of origin of the samples tested. However, positive statements about area differences must await analysis of a larger number of samples.

In addition to rabbit and chicken immunizations, extensive isoimmunizations

Table 5.--Demonstration of blood types in chum salmon using chicken antisockeye salmon sera.

Sera	Test cell sample number													
	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Serum 210:														
Absorbing cell														
No. 18	+	++++	+++	++	++++	++++	++++	0	+++	+	+++	+++	++++	+++
No. 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Serum 220:														
Absorbing cell														
No. 18	++	++++	++	++	++++	+++	+++	0	++	+	++	++++	+++	++
No. 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No. 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6.--Demonstration of blood groups in pink salmon

(Antiserum chicken E213 absorbed with red blood cells from Cordova pink salmon #6)

	Sashin Creek							Kodiak					Cordova
Sample number	1	2	3	4	5	6	7	1	3	4	5	9	6
Reaction	-	-	-	-	+	-	-	+	+	+	+	+	-

have been conducted on sockeye salmon (O. nerka). The fish used in all of these experiments were from the Columbia River (where this species is known as the blueback salmon). In 1955 and 1956, 20 to 30 adult salmon migrating up the Columbia River were captured and isoimmunized over periods of one to two months without any detectable development of isoimmune antibodies. Since many of these fish died before an adequate period for the production of isoimmune antibodies had elapsed, no conclusions about the antigenic heterogeneity of salmon could be drawn from these experiments.

In order to have fish available for isoimmunization over a longer period, a group of sockeye yearlings were obtained from the Winthrop Hatchery of the U. S. Fish and Wildlife Service in 1956 and reared in salt water at the Deception Pass Marine Research Station of the State of Washington Department of Fisheries. Isoimmunizations involving 100 fish, 50 pairs being cross-immunized, were started in August of 1957. During the period of this experiment the water temperature ranged from 14° C. to 9° C. with a mean of approximately 12° C.

Of these 100 fish, 15 produced isoimmune antibodies after periods of 7 to 14 weeks and 5 to 11 inoculations. Because of the small size of these salmon, due to their being reared in captivity, only small amounts of the isoimmune sera could be collected. In addition, the sera were of low titer, most reacting only to 1 in 4 dilution. Sufficient amounts of serum were obtained from seven of these fish to compare the specificity of the reactions against a number of individuals.

The results of these tests are presented in table 7. Inspection of these data reveals that all seven of the sera possessed different patterns of specificity, and at least eight different patterns of antigenic composition were shown by the cells tested. Thus, it would appear that the extent of antigenic variability in salmon is of the same order of magnitude as that found in other animals which have been extensively studied. Further study and application of this heterogeneity will depend on the availability of larger salmon, and experimental facilities for holding them.

DISCUSSION

The demonstration of blood group differences in four species of Pacific salmon, as outlined in this paper, along with the demonstration that, in some cases, these characters can serve as markers of racial identity, indicates the existence of valuable tools for the solution of many of the population problems encountered in the management and conservation of these important fishes. However, more research and developmental work must be done. One of the biggest problems is in the production of sufficient type-specific sera to test large numbers of individuals from populations of interest. In all of the immune sera we have produced in rabbits and chickens, most of the antibodies reacted with antigens possessed by all members of the species or genus. The production of type-specific sera in even small amounts required large quantities of cells for absorption.

It is interesting to note that several of the immune sera were capable of demonstrating blood group differences in a species other than the one used for immunization, but could not be used to demonstrate differences in the immunizing species. This would appear to indicate that related antigens are fixed in one species and segregating in another. These observations are somewhat analogous to antigenic relationships which have been demonstrated between man and Rhesus monkeys (Landsteiner and Weiner 1941), and recently between cattle and bison (Owen, Stormont and Irwin 1958), and between tahrs (Hemitragus jamlahicus) and a variety of other Artiodactyl species (Stormont and Suzuki 1958). Further extension of these observations in fishes may result in the production of type-specific sera in practical quantities.

Our isoimmunization experiments with sockeye salmon indicate considerable antigenic diversity exists in this species. Similar and more extensive studies have been conducted with rainbow trout and will be presented in another paper. As with other animals, isoimmunization appears to be the most promising approach to the problem of blood group differences in fishes. The species of Pacific salmon are particularly difficult animals on which to conduct isoimmunizations as they invariably die after becoming sexually mature at two to six or seven years of age. It is only shortly before their death (two weeks to three or four months) while they are on their spawning migration that they become readily available. They also grow quite slowly in captivity and only one species, the sockeye, can be readily held in fresh water throughout its lifetime. At least some of these difficulties can be overcome and we hope to continue our attempts to do so.

Table 7.--Comparison of the specificities of isoimmune sera produced in sockeye salmon.

	Serum						
Cells	14-4	14-6	14-10	15-1	22-10	24-2	13-2
13-1	0	0	+	+	+	+	+
14-3	+	0	0	0	0	+	0
14-5	0	+	0	+	0	0	0
14-9	0	0	+	+	0	+	0
15-1	-	+	+	0	-	0	0
15-7	0	0	0	-	-	-	0
22-9	-	-	-	-	+	+	0
22-10	+	+	0	+	0	-	-
24-1	+	+	0	+	-	+	0

+ Agglutination, 0 No agglutination, - Not tested.

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SUMMARY

1. Through the use of absorbed rabbit and chicken antisalmon-erythrocyte sera, blood types were demonstrated to occur in sockeye, chinook, chum, and pink salmon.

2. Some of these types appear to differ in their frequency of occurrence between different geographic races.

3. Isoimmunizations between individuals of race of sockeye salmon indicated the existence of at least eight different antigenic types or combination of types within this race.

4. The usefulness of this kind of research to fishery management and some of the problems involved in its practical utilization are discussed.

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